THE OXYGEN-CONSUMPTION OF COLPIDIUM CAMPYLUM

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Introduction

Interest in the respiratory exchange of Protozoa dates from the work of Vernon (1895) on the radiolarian, *Collozöum inerme*. In the forty-three years that have elapsed since the publication of Vernon's paper, observations have been made on the gas-metabolism of species belonging to three of the four major groups of unicellular animals. During this period, however, the development of protozoölogical technique has lagged behind the very rapid improvement in physiological and biochemical procedure, so that much of the data which have accumulated are difficult or impossible to evaluate.

Beginning with Lund's (1918*b*) work on *Paramecium caudatum*, oxygen-consumption of ciliate protozoa has repeatedly been found to be relatively unaffected by cyanide, suggesting that the mechanism by which these forms use molecular oxygen differs fundamentally from the usual one found in aerobic cells. The nature of the causes underlying this difference should prove to be of considerable general interest. It seemed worthwhile, therefore, to begin a careful study of the intracellular oxidative mechanism in a species of ciliate. The holotrichous form *Colpidium campylum* was chosen for the purposes of this investigation. In order critically to study the respiratory mechanism, it was necessary to have precise measurements of the normal rate of oxygen-consumption. The existing data provide no such sound basis for further experimental work with this species. Accordingly, the rate of oxygen-consumption of *C. campylum* has been determined manometrically under carefully controlled and, therefore, reproducible conditions.

The current paper is a contribution to the growing literature in a relatively new field of investigation dealing quantitatively with the biochemical properties of pure suspensions of protozoa. Invaluable data have resulted from the application of such methods of study in the field of bacteriology. Their extension to the Protozoa, although highly desirable in view of the biological and medical importance of this phylum, was formerly rendered impracticable by the complexity of the media required for cultivation of most protozoan forms. With the develop-

ment of suitable isolation methods and of artificial media capable of supporting growth, a limited number of species are now available in bacteriologically sterile, non-particulate cultures. Such cultures offer material for biochemical investigation which is more homogeneous and free from specialization of cellular function than metazoan tissue slices.

This research was carried out under the supervision of Professor Gary N. Calkins of the Zoölogy Department of Columbia University. The author wishes to express his appreciation of the interest which Professor Calkins has maintained in the progress of the work, and to acknowledge his indebtedness to him for his aid in preparing the manuscript.

Material and Methods

A bacteria-free culture of *Colpidium campylum* (Stokes) Breslau was obtained from the Lenox Hill Hospital through the courtesy of S. M. Nagy. This strain was originally isolated by Hetherington and identified by Lwoff. Cultures of the ciliates were grown in 20×150 mm. cotton-plugged Pyrex tubes containing 15 cc. of sterile medium. Transfers were made with sterile volumetric pipettes of 1 ml. capacity, observing the usual precautions in order to prevent bacterial contamination. The medium employed for cultivation of the organisms consisted in a 2 per cent solution of tryptone (Difco) containing 0.1 per cent yeast-extract (Difco), made up in the following salt solution:

KH_2PO_4 (Sørensen)	0.5 gram
NaCl	0.4 "
$MgSO_4.8H_2O$	0.1 "
$Ca(NO_3)_2.4H_2O$	0.1 "
Distilled water	1,000 cc.

The reaction of the medium after autoclaving was pH 6.6 ± 0.02. The pH-measurements were made with a quinhydrone electrode connected through a saturated KCl-agar bridge to a saturated KCl-calomel half-cell.

The experimental suspensions were made up with material from cultures incubated 48 hours in a thermostat-controlled room at 23° C. The supernatant layer of actively swimming ciliates was poured off the top of a culture into a clean centrifuge tube, being careful not to stir up the precipitate of dead organisms at the bottom. Material from several cultures was concentrated by centrifuging and transferring to a single clean test-tube. Suspensions prepared in this manner cannot be described as absolutely bacteria-free, since the cultures from which they are made up undoubtedly become contaminated as soon as they are exposed directly to the air. For practical purposes, however, such sus-

pensions may be regarded as bacteria-free, since the number of bacteria present even after several hours is entirely negligible in comparison with the magnitude of the protozoan population.

In the present investigation the rate of oxygen-consumption by *Colpidium campylum* is stated in terms of the number of cubic millimeters of gas, reduced to 0° C. and 760 mm. pressure, taken up per million cells. When results are expressed in this manner, their accuracy is largely determined by the precision with which the number of cells in the experimental suspensions is estimated. The following procedure has been found satisfactory. By means of a calibrated pipette, samples were withdrawn from the concentrated suspension alternately to be used in performing the measurements and in estimating the number of cells. The pipette used in taking samples delivered 0.995 cc. at room temperature. Before taking each sample, the tube containing the concentrated suspension was rolled vigorously between the palms of the hands or flipped carefully to insure uniform distribution of the contents. Before any samples were taken the pipette was first wet by drawing up some of the suspension. This procedure helps to correct for the tendency of the cells to stick to the sides of the pipette. As a rule, two samples were transferred into the main chamber of each manometer-vessel. The samples to be used in determining the concentration of the suspension were placed in clean test-tubes containing 5 drops of Bouin's alcoholic fixative. Each tube received a single 0.995 cc. sample; 9 cc. of distilled water were added from a calibrated burette, and the tubes were plugged with cotton and set aside in the refrigerator. When counts were to be made, the tubes were removed and shaken vigorously. Single samples from each were placed in separate clean test-tubes, employing the same pipette which was used in taking the original samples from the experimental suspensions. The contents of each of these tubes were diluted with 9 cc. of distilled water from the calibrated burette, and counts were made on 0.995 cc. samples withdrawn from the resulting suspensions after thorough shaking. A Sedgwick-Rafter counting chamber of the circular type was used. In order to facilitate counting, the bottom surface of the chamber was ruled off into 5 mm. squares with a diamond-point pencil. The number of ciliates in the entire contents of the chamber was counted under a binocular dissecting microscope, using the 2.3 × objective and 9 × oculars. The results of the several counts were averaged and multiplied by 100 to obtain the average number of cells in each sample taken from the original concentrated suspension. This figure, multiplied by the proper small integer representing the number of samples placed in each manometer vessel and expressed as a decimal part of one million, was used in calculating

the rate of oxygen-consumption. The difference between the counts obtained with individual samples and the average for all samples taken from the same original suspension was taken as an index of the probable error of counting. From the differences obtained in this way in each experiment the average percentage difference between counts performed on samples from the same suspension was calculated. This constant is a mixed one including both the sampling error and the error of counting.

Oxygen consumption was measured directly, using Warburg manometers fitted with rectangular vessels similar to those employed by Warburg, Kubowitz and Christian (1931), but with an inset chamber for potassium hydroxide. The theory of the method has been adequately summarized and appraised by Dixon (1934). Hence, only those details will be discussed here which are of particular importance in connection with this investigation. In the majority of the experiments four or five manometers were used, one of these in each case serving as a thermo-barometric control. The average capacity of the manometer vessels was 8.812 cc. to the level of the manometric fluid (Brodie's solution colored with Sudan III), and the average volume of the gas space was 6.689 cc. The manometer constants were calculated by substituting in the formula derived by Warburg. The total volume of oxygen consumed was obtained from the algebraic difference between the observed change on the scale of the experimental manometer and that on the scale of the thermobarometer by multiplying by the proper manometer constant. The rate of oxygen-consumption was calculated as the quotient obtained by dividing the total volume of oxygen consumed in unit time (1 hour) by the total number of cells in the suspension expressed as a decimal part of one million. In order to insure efficient absorption of the carbon-dioxide evolved during respiration, small rolls of No. 40 Whatman (starch-free) filter paper were placed in the inset chambers of the vessels and soaked with 20 per cent carbonate-free KOH. The volume of alkali added was 0.133 cc. in every case. The temperature was controlled by immersing the vessels in a water bath at $19.8° \pm 0.02°$ C. The manometers were attached to a shaking device and shaken constantly through 5 cm. of arc, except during the time necessary for taking readings.

Experimental Results

Dixon and Elliott (1930) have shown that in performing manometric measurements, if the results are to represent the true gaseous exchange of the tissue or cell-suspension, certain purely mechanical factors must be taken into consideration. Under certain circumstances the capacity

of the manometer vessels and their shape, together with the rate at which the manometers are shaken, may determine the accuracy of the measurements. The manometers should be shaken rapidly enough to render oxygen-consumption of the contained tissue or cell-suspension independent of the diffusion equilibrium between the gas space and the fluid bathing the tissue or cells.

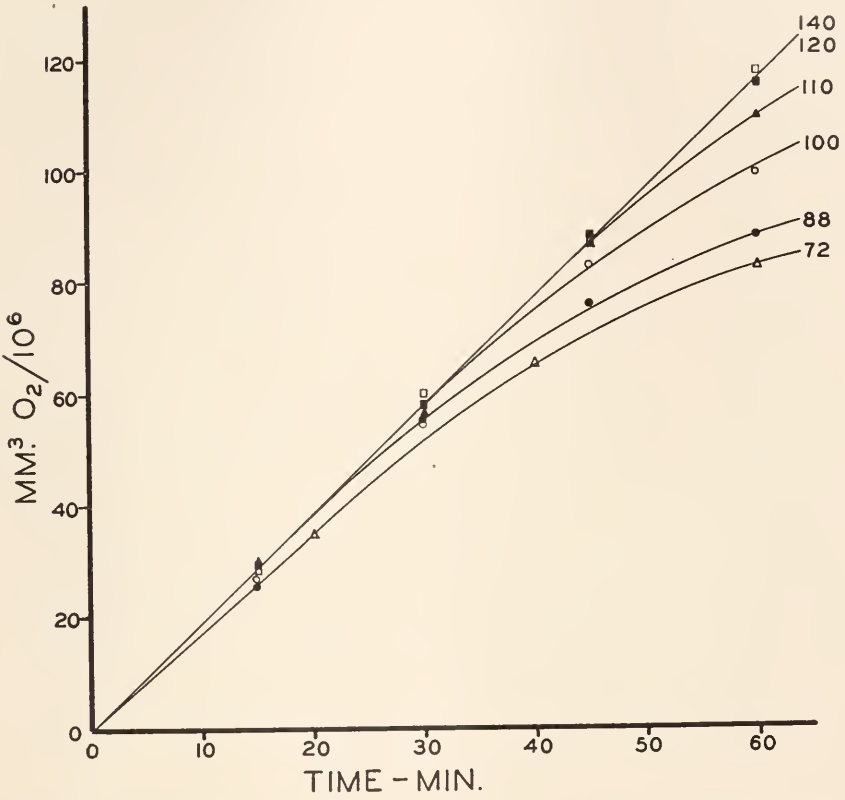

FIG. 1. Oxygen-consumption by suspensions of *C. campylum* at different rates of shaking. Ordinate—cu. mm. of O_2 per million protozoa; abscissa—time in minutes. The figures at the right, opposite the curves, refer to the rate of shaking expressed as the number of complete cycles per minute.

At the beginning of the present investigation, therefore, a series of experiments was carried out with the object of finding a suitable rate of shaking for suspensions of the proper concentration to insure obtaining significant readings. The results are shown in Fig. 1. Each point represents the average of the data obtained with three different manometers except for the experiment in which the rate of shaking was

140 cycles per minute, where an unfortunate accident eliminated two of the manometers. The average number of cells in the suspensions was about 250,000. From the data obtained it is clear that with suspensions of the order of density of a quarter of a million cells the rate

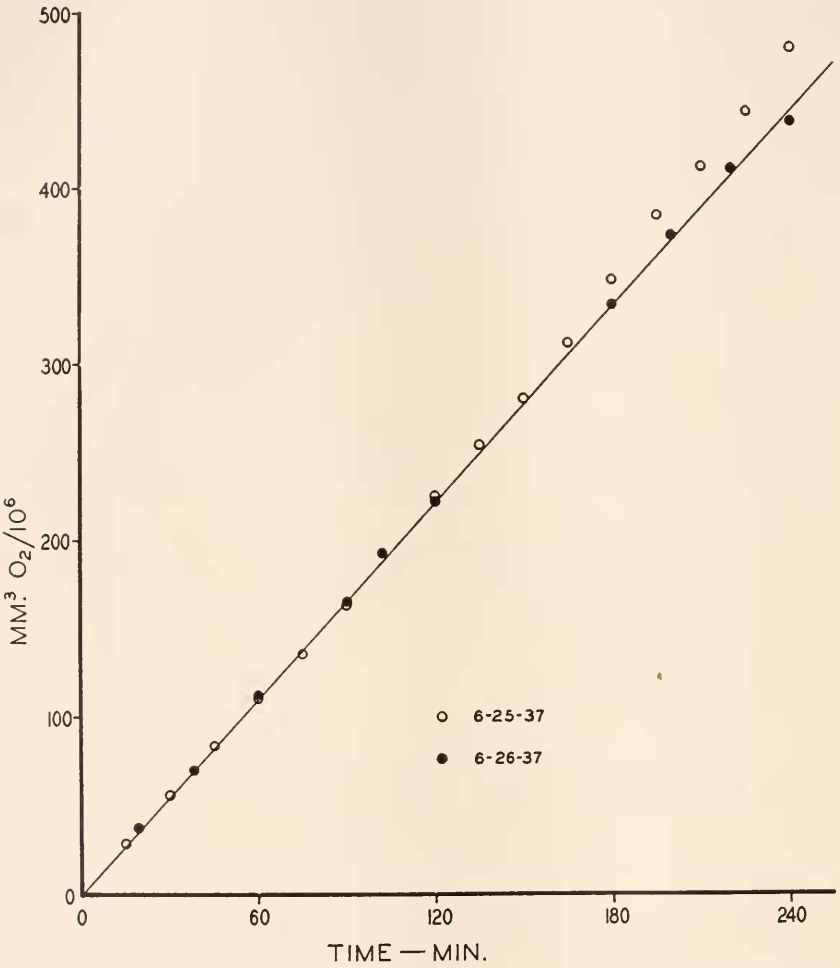

FIG. 2. Oxygen-consumption by suspensions of *C. campylum*. Data of single experiments performed with the same manometer. Ordinate—cu. mm. of O_2 per million protozoa; abscissa—time in minutes. Explanation in text.

of oxygen-uptake is dependent on the rate of shaking until a frequency of about 120 cycles per minute is attained. Accordingly, in all subsequent experiments the manometers were shaken at a rate of 120 cycles per minute.

In Fig. 2 are shown the results of two experiments, performed on successive days with the same manometer, in which the oxygen-consumption of suspensions of *Colpidium campylum* was followed during a period of over four hours. The duration of individual runs was one hour. The concentrations of the experimental suspensions were approximately the same, about 230,000 cells in each case. The open circles in Fig. 2 represent the data obtained June 25, 1937. In this experiment tap-water was used in the vessel of the manometer which served as a thermobarometric control. A gradual increase in the rate of oxygen-consumption is indicated after the second hour. Since the increase was very gradual, it seemed likely that it might be only an apparent one brought about through the cumulative effects of autoxidizable substances and bacterial contamination in the suspension, or by differences between the solubilities of the respiratory gases in tap-water and in culture fluid. All of these effects could readily be compensated by using fresh culture medium in the vessel of the thermobarometer. This was done in the experiment performed the following day, the data of which are represented by the filled circles in Fig. 2. It is evident that when the proper precautions are taken in setting up an experiment the observed rate of oxygen-consumption by suspensions of *C. campylum* remains constant over a period of at least four hours. The average rate computed from the data of June 26 is 111.0 mm.^{3} $\overline{O}_2$/hr./million.

In order to obtain critical measurements of the rate of oxygen-consumption, a series of experiments was performed in which three or four samples were tested in each run. A summary of these experiments is given in Table I. By averaging the results of the 36 measurements included in the table, a value of 112.5 mm.^{3} O_2/hr./10^6 is obtained for the rate of oxygen-consumption by suspensions of *Colpidium campylum* in the medium employed for their cultivation. Comparison of this figure with that of 111.0 mm.^{3} $\overline{O}_2$/hr./10^6 given in the preceding paragraph shows that individual runs yield data of good precision.

The data of Table I provide an index of the accuracy with which samples of like density can be taken from the same original concentrated suspension, using the technique described in this paper. The standard deviation between the oxygen-quotients obtained for different samples in the same run was 0.0194. Since the combined error of counting and sampling is of the order of about 4 per cent, it is evident that the sampling error falls well within the limits of the general method of relating the volume of oxygen consumed to the number of respiring cells. The data show that no significant difference in results

is obtained with different manometers provided the latter are accurately calibrated. While the average result obtained with manometers Nos. 203 and 205 was higher than with the other two manometers, the difference does not appear consistently and represents only about 1.2 per cent of the average hourly uptake, whereas for the 36 measurements included in the table the standard deviation of the average $Q_{\bar{O}_2}$-value is 0.0363. In two of the experiments included in Table I it will be noted

TABLE I

Summary of the data of eleven experiments in which three or more samples were tested simultaneously. Duration of each run = 1 hour. Temperature = 19.8° ± 0.02° C. Gas-mixture — air. Rate of shaking = 120 cycles/minute.

Date	Hour	No. of cells × 10^{-6}	$Q_{\bar{O}_2}$ — mm.³ $\bar{O}_2$/hr./10^6				
			Manometer number				Average
			200	202	203	205	
6–2	1	0.226	109.4	106.2	111.1		108.9
	2		110.3	103.0	111.9		108.4
6–5	1	0.139		117.5	116.0	113.8	115.7
	2			118.8	118.4	119.7	119.0
6–21	1	0.238	116.0		113.9	117.4	115.7
7–1	1	0.247	111.2		110.2	112.0	111.1
	2		109.2		110.4	106.4	108.7
7–7	1	0.321	111.3		113.4	121.3	115.3
7–14	1	0.234	106.8	110.3			108.5
		0.466			108.9	109.0	109.0
7–21	1	0.251	116.7	113.0			114.9
		0.376			113.9	109.3	111.6
	2	0.251	114.9	114.1			114.5
		0.376			114.4	110.1	112.3
Averages		0.284	111.8	111.8	113.0	113.2	*112.5*

that suspensions of apparently greater density were used in manometers Nos. 203 and 205 than in the other two manometers in the same runs. In preparing these experiments, different volumes of the original concentrated suspension were measured into the vessels of the respective manometers. The total volume of the suspension, and, hence, the surface-volume relationship, were thus varied without altering the actual density of the suspensions. This was done in order further to check

whether the rate of shaking (120 cycles per minute) was adequate to insure uniform results when larger populations were to be tested. The data do not provide any evidence to the contrary.

Discussion

Comparison of the data presented above with those of earlier workers is difficult because of the variety of conditions, often imperfectly known, under which previous measurements have been carried out. Table II is a summary of the results obtained by other investigators where comparable procedures have been employed and where the data could be expressed in a standard way as the volume of oxygen in cubic millimeters consumed per million cells per hour.

Investigations have ranged in exactness from observations on the ability to survive under conditions of anaerobiosis (Pütter, 1905) to determination of the gaseous exchange of single organisms in a capillary micro-respirometer (Kalmus, 1927, 1928; Howland and Bernstein, 1931). Efforts toward quantitative measurements began, although they attained no accuracy, with the use of absorption apparatus (Barratt, 1905) and various modifications of the Winkler technique (Wachendorff, 1912; Lund, 1918). Von Fenyvessy and Reiner (1924), Soule (1925) and Amberson (1928) probably obtained more accurate results by carefully analyzing samples of gas from sealed cultures, using the Barcroft-Haldane-Henderson apparatus. Unfortunately, none of these investigators attempted to express his findings in quantitative terms. Beginning with the experiments of Błedowski and Zweibaum (1915) the majority of studies have involved the use of manometric methods.

The earliest measurements were made on material from mixed cultures in media of uncertain composition. The disadvantages of such cultures have been discussed elsewhere (Jahn, 1931; Phelps, 1931, 1935; Raffel, 1932) in connection with growth studies. In attempting to measure respiration of protozoa in mixed suspensions, particular difficulties are encountered, since the metabolism of the bacteria or other foreign microörganisms may alter the results directly to an indeterminate extent. The importance of this factor is brought out by comparison of the following data. Cook and Haldane (1931) have shown that at 16° C. *B. coli communis* consume 17.5 mm.^{3} $\overline{O}_2$/hr./mg. bacterial nitrogen in the absence of any oxidizable substrate. Since these bacteria contain about 8.3 per cent of their dry weight of nitrogen, the average basal oxygen-uptake is 1.5 mm.^{3} $\overline{O}_2$/hr./mg. dry weight. ($\underline{Q}\bar{o}_2$ is obtained by multiplying by 0.083—not by dividing, as stated by Cook

and Haldane—and is of the same order of magnitude as for yeast or mammalian tissues.) In the presence of an oxidizable substrate, *c.g.*, lactate, $Q\bar{O}_2$ becomes 12 mm.^{3} $\bar{O}_2$/hr./mg. dry weight. When *B.*

TABLE II

Summary of the Results of other Investigators

Species	Investigator	Temp.	$Q\bar{O}_2$	Remarks
		°C.		
Actinosphaerium eichhorni...	Howland, 1931	20.0?	1130.0	Bacteria present
Chilomonas paramecium.....	Mast, Pace and Mast, 1936	25.0	16.4	Bacteria-free suspensions
Colpidium campylum.....	Pitts, 1932	24.0	151.5	Bacteria for food
C. colpoda.....	Wachendorff, 1912	17.0	191.0	1 day culture
			151.0	10 day culture
			59.0	30 day culture
		7.0	33.0	
C. colpoda.....	Peters, 1929	25.0?	200.0	Bacteria-free suspensions?
Colpoda sp.....	Adolph, 1929	19.8	575.0	Clone "B"
			537.5	Clone "C"
			1185.0	Clone "E"
Paramecium caudatum.....	Lund, 1918	25.0	140.0	Fed
			40.0	Starving
	Zweibaum, 1922	23.0	737.0	Before conjugation
			3481.0	During conjugation
			2142.0	After conjugation
	Necheles, 1924		3850.0	
	Kalmus, 1927	21.0	5200.0	Single cells
	1928	17.0	4600.0	Single cells
		13.0	3300.0	Single cells
Paramecium caudatum.....	Howland, 1931	20.0	490.0	Single cells
P. multimicronucleatum.....	Mast, Pace and Mast, 1936	25.0	1021.0	Bacteria present
Spirostomum ambiguum.....	Specht, 1935	25.0	2590.0	

coli oxidizes formate, the rate of $\bar{O}_2$-uptake is about 40 mm.^{3} $\bar{O}_2$/hr./mg. dry weight of bacterial suspension. The holotrichous ciliate *Glaucoma pyriformis* consumes on an average about 35 mm.^{3} $\bar{O}_2$/hr./mg. dry

weight at 22° C. in 2 per cent peptone-water (M. Lwoff, 1934). In order to overcome the disadvantages of mixed suspensions, recourse has been made in the past to diminishing the number of bacteria, etc., by washing (Wachendorff, 1912; Necheles, 1924; Adolph, 1929; Emerson, 1930; Specht, 1935), or to balancing the respiration of the mixed population against that of a suspension of the bacteria alone (Zweibaum, 1921; Grobicka and Wasielewska, 1925; Gerard and Hyman, 1931). Bacteria-free cultures of protozoa were first employed in measurements of respiration by Soule (1925).

In most previous investigations no atttempt has been made to control the reaction of the medium in which the organisms were suspended, and the relation between pH and the rate of oxygen-consumption is not known. In only a few instances has the age of the population been taken into consideration, although the importance of this factor was early recognized (Wachendorff, 1912). Phelps (1935, 1936) has shown that growth of *Glaucoma pyriformis* in bacteria-free cultures resembles that of yeast, Protophyta, and bacteria. There is a logarithmic phase during which the population increases at a constant rate, a phase of negative acceleration when the rate of increase in the population decreases although the total number of cells continues to increase, and a stationary state in which the death rate just balances the rate of increase. The suspensions of *Colpidium campylum* employed in the present investigation were made up with material from cultures in the stationary growth phase. It is reasonable to suppose that significantly different results might have been secured with material from cultures in the logarithmic phase or that of negative acceleration of growth.

There are no reliable data correlating the oxygen-consumption of protozoa with temperature, although measurements have been made at temperatures ranging from 6.5° to 28° C. Wachendorff's (1912) results indicate that the rate of respiration of *Colpidium colpoda* is quadrupled by an increase of 10° C. in temperature. From the results of experiments with single *Paramecium caudatum* at 13°, 17°, and 22° C., Kalmus (1928) concluded: "Wie die bisher untersuchten messbaren Lebensvorgänge des Objekts, folgt auch die Atmung in der Umgebung des Temperatur-optimums der RGT-Regel."

Wachendorff (1912) and Lund (1918) employed the Winkler technique for determining oxygen-consumption. In spite of the fact that this method is hardly adapted for the precise estimation of minute changes in oxygen-tension, the measurements were made with suspensions of only a few thousand cells. In order to obtain significant readings it was necessary to prolong the experiments over a period of 6–24 hours, during which interval the increase in bacterial flora must

have been sufficient to influence the results markedly even if the protozoa themselves failed to divide. The work of Necheles (1924), Adolph (1929) and Specht (1935) is subject to criticism of the same sort. These investigators have used manometric methods in measuring the oxygen-consumption of mixed suspensions containing between 900 and 12,000 cells. With suspensions of such concentrations measurable changes are produced only after relatively long periods of time. Consider the data obtained in the present investigation. At the rate of 112.5 mm.^{3} O_2/hr./million, 5,000 cells would use 0.5625 mm.^{3} of oxygen in an hour. Since the average value of the gas constant for the manometers employed in this research was 0.661, the average change produced on the manometer scale by 5,000 cells in one hour would be about 0.836 mm. With the aid of a hand-lens, readings can be made to the nearest 0.1 mm.; hence, the error introduced in such measurements in taking readings alone would be of the order of magnitude of 12.0 per cent.

The first measurements of the oxygen-consumption of Protozoa made under satisfactory conditions are those of A. Lwoff (1933). These experiments were done at 28° C. with suspensions of *Strigomonas fasciculata, S. oncopeltis* and *Leptomonas ctenocephali* from 96-hour bacteria-free cultures in 2 per cent peptone solution at pH 7.0. The data obtained by M. Lwoff (1934) for *Glaucoma pyriformis* are the first dealing with the oxygen-consumption of a ciliate under adequately controlled conditions. Unfortunately, the Lwoffs have expressed their findings in terms of the volumes of oxygen consumed per milligram dry weight of the suspension, so that it is impossible to compare their results with those either of the current research, or of other investigators.

Summary

A procedure has been described for obtaining accurate measurements of the rate of oxygen-consumption of Protozoa. This procedure involves the use of Warburg manometers and concentrated suspensions of organisms made up from bacteria-free cultures. The rate of oxygen-uptake by the holotrichous ciliate *Colpidium campylum* has been determined. The average of 36 measurements gave a value of 112.5 mm.^{3} $\bar{O}_2$/hr./million for the rate at 19.8° C. in the medium in which the infusoria had been growing for 48 hours. The rate remained constant over a period of more than four hours. The standard deviation of the measurements was 0.0363.

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